JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

Stereochemistry and Mechanism of a Microbial Phenylalanine Aminomutase

Nishanka Dilini Ratnayake,† Udayanga Wanninayake,† James H. Geiger,† and Kevin D. Walker*,†,‡

†Department of Chemistry and ‡Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824, United States

Supporting Information

ABSTRACT: The stereochemistry of a phenylalanine aminomutase (PAM) on the andrimid biosynthetic pathway in *Pantoea agglomerans* (*Pa*) is reported. *Pa*PAM is a member of the 4-methylidene-1*H*-imidazol-5(4*H*)-one (MIO)-dependent family of catalysts and isomerizes (2*S*)-α-phenylalanine to (3*S*)-*β*-phenylalanine, which is the enantiomer of the product made by the mechanistically similar aminomutase *Tc*PAM from *Taxus* plants. The NH_2 and *pro*-(3*S*) hydrogen groups at C_α and C_β, respectively, of the substrate are removed and interchanged completely intramolecularly with inversion of configuration at the migration centers to form *β*-phenylalanine. This is a contrast to the retention of configuration mechanism followed by *Tc*PAM.

Pantoea agglomerans (*Pa*) bacteria produce the antibiotic andrimid, a polyketide/non-ribosomal peptide that inhibits the bacterial acetyl coenzyme A carboxylase. One of the building blocks used during andrimid assembly is (3*S*)-*β*-phenylalanine, derived by isomerization of (2*S*)-α-phenylalanine via the AdmH catalytic domain, that functions as a phenylalanine aminomutase (PAM)[1] (designated herein as *Pa*PAM). The primary amino acid sequence of *Pa*PAM is homologous to a class I lyase-like family of catalysts (comprised of ammonia lyases[2−4] and aminomutases[1,5−7]) that contain a signature 4-methylidene-1*H*-imidazol-5(4*H*)-one (MIO) prosthesis. The biosynthetic pathway to andrimid continues through the AdmJ catalytic domain that adenylates (3*S*)-*β*-phenylalanine and then transfers the activated intermediate to a free-standing thiolation domain, AdmI. Subsequent octatrienoylation, amidation, and ketonization of the *β*-amino acid by other enzymatic domains on the assembly line produce the final product[1] (Scheme 1). Herein, we report on the characteristics of the mechanism and stereochemistry of the *Pa*PAM reaction.

AdmH was cloned and expressed as an N-terminal His_6 fusion from the pET-24b(+) vector in *Escherichia coli* (BL21). Activity assays with expressed *Pa*PAM and (2*S*)-α-phenylalanine confirmed the product as (3*S*)-*β*-phenylalanine, after the amino acids in the reaction mixture were converted to their *N*-(1(*S*)-camphanoyl) methyl esters. The derivatized *β*-amino acid was identified by GC/EI-MS analysis to have a retention time identical to that of authentic *N*-[(1′*S*)-camphanoyl]-(3*S*)-*β*-phenylalanine methyl ester and not the 1′*S*,3*R*-isomer.

To assess the mechanism and cryptic stereochemistry of the proton transfer of the *Pa*PAM reaction, a mixture of [ring,

Scheme 1. Biosynthetic Pathway to Andrimid

(*S*)-α-Phenylalanine
AdmH
(*S*)-β-Phenylalanine
AdmJ
ATP PPi
(*S*)-β-Phe-AMP
several steps
Andrimid

β-C-$^2H_6$]-(*E*)-cinnamate (**1**) and [2-^{15}N]-(2*S*)-α-phenylalanine (**2**) (each 98+% enriched), a mixture of isotopomers unlabeled (**4**) and [U-^{13}C,2-^{15}N]-(2*S*)-α-phenylalanine (**5**) (98+% enriched), racemate [ring,3-$^2H_6$]-(2*R*,3*S*)/(2*S*,3*R*)-α-phenylalanine (**8**/**9**) (98+% enriched), and [ring,2,3-$^2H_7$]-(2*S*,3*S*)-α-phenylalanine (**11**) (90% ee, 98+% enriched) were separately incubated with the aminomutase. The resulting *β*-phenylalanine isotopomers were derivatized to their *N*-benzoyl methyl esters and analyzed by GC/EI-MS; derivatized unlabeled *β*-phenylalanine was identically analyzed.

Based on the reaction of the homologous PAM from *Taxus* plants, (*E*)-cinnamate is a proposed intermediate of the forward reaction during isomerization of α- to *β*-phenylalanine and is reported as a substrate (at pH 10.5 in 6 M NH_4^+ salt) used to make a mixture of α- and *β*-phenylalanines.[8] Thus, to evaluate whether *Pa*PAM could transfer the amino group from phenylalanine to an exogenous (*E*)-cinnamate, a mixture of **1** and **2** was incubated with *Pa*PAM. The reactions were quenched, and the amino acids were derivatized and analyzed by GC/EI-MS. Analysis of the fragment ions suggested that the *β*-phenylalanine (>99%) was ^{15}N-enriched (**3**) with no unlabeled isotopomer present and was not derived from [ring,*β*-C-$^2H_6$]-(*E*)-cinnamate (Table 1A). The mode of intramolecular exchange was further confirmed by incubating the aminomutase with a mixture of **4A** and **5**. The *β*-amino acid derivatives were analyzed as before, and the ^{14}N and ^{15}N of the *β*-amino acid products (**6A** and **7**, respectively) remained coupled to the carbon scaffold of the corresponding α-amino acid substrate (Table 1B).

An α-phenylalanine racemate contained the nonproductive (2*R*)-enantiomer **8** and the productive (2*S*)-enantiomer **9**; the reaction stereospecificity was assessed previously by incubating

Received: April 4, 2011
Published: May 11, 2011

Table 1. β-Phenylalanine Isotopes Derived from *Pa*PAM Catalysis and Various Labeled α-Phenylalanines

	Substrate: α-Phenylalanine Isotope	Biosynthesized β-Phenylalanine Isotopes
A	**1** (^{2}H, 98%); **2** (^{15}N, 98%)	**3**
B	**4A**; **5** (•) = ^{13}C, 98%	**6A**; **7**
C	**8** (^{2}H, 98%); **9** (^{2}H, 98%)	**10**
D	**11**	**12**
E	**13**	**14**

Scheme 2. Reaction Catalyzed by *Pa*PAM Proceeds with Inversion of Configuration

(2*S*)-α-Phenylalanine → *Pa*PAM → (3*S*)-β-Phenylalanine

*Pa*PAM (2 mg) separately with unlabeled (2*S*)- and (2*R*)-α-phenylalanine (data not shown). The β-amino acid derivatives analyzed by GC/EI-MS indicated that the deuterium at the *pro*-(3*R*) position of the substrate was retained in the (3*R*)-β-phenylalanine product (**10**) (Table 1C). In contrast, the same analysis of the derivatized β-phenylalanine isotopomer isolated after incubation of *Pa*PAM with **11** indicated that no deuterium remained at C3 of the product **12** (Table 1D). All of the deuterium at the *pro*-(3*S*) migrated to C2, suggesting that this migration was completely intramolecular without exchange with solvent protons. This latter observation supported an earlier study that showed all three of the deuteriums of [$^2H_8$]-α-phenylalanine, after incubation with *Pa*PAM, were completely retained in the β-amino acid.[9]

The stereochemistry of the hydrogen rebound during the aminomutase reaction was evaluated by incubating [3,3-$^2H_2$]-α-phenylalanine (**13**) with *Pa*PAM for 2 h. Thereafter, the amino acid isotopomers (including **14**, Table 1E) were derivatized as their *N*-acetyl methyl esters and dissolved in $CHCl_3$ for ^{2}H NMR analysis.

The ^{2}H NMR profile of the derivatized [^{2}H]-labeled amino acid mixture contained peaks at δ 5.40 (NC_βD) and 2.83 (HC_αD), which were identical to those of the authentic racemate [2,3-$^2H_2$]-*N*-acetyl-(2*S*,3*R*)/(2*R*,3*S*)-β-phenylalanine methyl ester.[5] These NMR data coupled with the known (3*S*)-stereochemistry[1] (also found herein) of the biosynthetically derived [^{2}H]-β-phenylalanine product established the biosynthetic product as the (2*R*,3*S*)-enantiomer. Further, the integrals of the peak areas (set to 1.0 deuterium) for the resonance signals at δ 5.40 (C_βD) and 2.83 (C_αD) were equal, suggesting that no hydrogen exchange occurs with the buffer protons during the isomerization, as shown earlier.[1,9] This contrasts the significant hydrogen exchange (∼60%) observed over 1 h with another MIO-dependent aminomutase from *Taxus* plants (*Tc*PAM)[5] and suggests that the *Pa*PAM active site excludes water more effectively. In addition, the ^{2}H NMR data strongly support a mechanism where *Pa*PAM shuttles the *pro*-(3*S*) hydrogen from C3 of (2*S*)-α-phenylalanine to C2 of the β-isomer product with inversion of configuration, while the amino group reattaches at C3, also with stereochemical inversion (Scheme 2). This is distinctly different than the retention of configuration mechanism of the *Tc*PAM reaction.[10]

As a member of the MIO-based aminomutases, *Pa*PAM likely proceeds through a stepwise mechanism where the migratory hydrogen and amino group are eliminated heterolytically from the substrate and held by the enzyme.[11] An ensuing *trans*-acrylate intermediate is proposed to provide the carbon skeleton upon which the amino group and hydrogen rebound with inversion of configuration.

To invoke the proper stereochemistry, *Pa*PAM likely removes and reattaches the amino and hydrogen groups to the same face of the cinnamate intermediate (**16A**) from which they originated (Scheme 3), whereas *Tc*PAM is proposed to remove and reattach the amino and hydrogen groups to the opposite face from which they originated (cf. **18A**), possibly via rotation of the intermediate in the active site[10] (Scheme 3). More importantly, the cinnamate intermediate on both pathways (cf. Scheme 3) is retained in the active site throughout the course of each isomerization reaction, as evidenced herein by incubating *Pa*PAM with **1** and **2** to make **3** exclusively. A similar assay with *Tc*PAM in a previous study showed essentially the same results.[10]

Evidence to support the proposed pathways for the *Pa*PAM and *Tc*PAM reactions was provided by incubating 2′-methyl-(2*S*)-α-phenylalanine (**15B**, 1 mM) at 31 °C for 1 h separately with each enzyme. The reactions were acidified, and the cinnamic acid analogues were separately extracted into ethyl acetate and converted to their methyl esters. The remaining aqueous fractions were basified, and the amino acids were derivatized to their ethyl carbamates with ethyl chloroformate, acidified, extracted into ethyl acetate, and finally methyl esterified. An aliquot of each extract was separately analyzed by GC/EI-MS. The distribution of 2′-methyl-β-phenylalanine and 2′-methyl-(*E*)-cinnamate made from 2′-methyl-(2*S*)-α-phenylalanine by *Pa*PAM catalysis was 98:2, while a reciprocal distribution (∼1:99) was observed for *Tc*PAM catalysis. Comparison of the kinetic parameters for *Pa*PAM (k_{cat} = 0.061 s^{-1}, β-amino acid production; K_M = 0.05 mM) and *Tc*PAM (k_{cat} = 0.002 s^{-1}, cinnamate production; K_M = 0.01 mM) with 2′-methyl-(2*S*)-α-phenylalanine showed that the catalytic efficiency (k_{cat}/K_M = 1.2 s^{-1} mM^{-1}) of *Pa*PAM was 6-fold greater than the efficiency of *Tc*PAM, due largely to the superior k_{cat}.

Scheme 3. *Route A*: Proposed Course of *Pa*PAM Reaction from (2*S*)-α-Phenylalanine to (3*S*)-β-Phenylalanine; *Route B*: Proposed Course of *Tc*PAM from (2*S*)-α-Phenylalanine to (3*R*)-β-Phenylalanine[a] and Proposed Rotamers of 2′-Methylcinnamate in the *Tc*PAM Reaction[a]

[a] The p-orbitals on the C═C and the ring are provided for perspective only and not intended to demonstrate molecular orbital concepts.

*Tc*PAM is proposed to access a second rotamer (**18A**)[10] from **16A** to make β-phenylalanine, involving rotations about the C_1-C_α and $C_{ipso}-C_\beta$ bonds. The 2′-methyl substituent of **15B** likely affected the rotation of **16B** to **18B**, and thus only **16B** was made by *Tc*PAM. In contrast, *Pa*PAM presumably utilized a single 2′-methylcinnamate rotamer (**16B**) to make 2′-methyl-(3*S*)-β-phenylalanine without encountering the torsional barrier. The difference in product distribution between the two enzymes supports a model consistent with steric and torsional strain between the 2′-methyl substituent and the C_α-hydrogen of the intermediary 2′-methyl-(*E*)-cinnamate in the *Tc*PAM reaction (cf. rotamer **18B** in Scheme 3). Notably, alternative or additive steric interactions between the 2′-methyl substrate and active-site residues can also prevent interchange between **16B** and **18B** and abort the *Tc*PAM reaction (Scheme 3). Conversely, it can be imagined that the single rotamer **16B** on the *Pa*PAM pathway need not encounter the same active-site interactions to proceed from 2′-methyl-(2*S*)-α- (**15B**) to 2′-methyl-(3*S*)-β-phenylalanine (**6B**).

The underlying mechanism responsible for the proposed rotational dynamics of *Pa*PAM and *Tc*PAM is not fully understood and is intriguing since the positions of most of the catalytic amino acids, the presumed H-bonding residues, and van der Waals interactions in each active site are conserved.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Materials, gas chromatography and mass spectrometry data, NMR data, experimental methods, and instrumentation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
walke284@msu.edu

■ ACKNOWLEDGMENT

The authors thank Christopher Walsh (Harvard Medical School) for providing the AdmH clone. This work was supported by the National Science Foundation (CAREER Award 0746432, K.D.W.).

■ REFERENCES

(1) Magarvey, N. A.; Fortin, P. D.; Thomas, P. M.; Kelleher, N. L.; Walsh, C. T. *ACS Chem. Biol.* **2008**, *3*, 542–554.
(2) Röther, D.; Poppe, L.; Viergutz, S.; Langer, B.; Rétey, J. *Eur. J. Biochem.* **2001**, *268*, 6011–6019.
(3) Asano, Y.; Kato, Y.; Levy, C.; Baker, P.; Rice, D. *Biocatal. Biotransform.* **2004**, *22*, 133–138.
(4) Rettig, M.; Sigrist, A.; Rétey, J. *Helv. Chim. Acta* **2000**, *83*, 2246–2265.
(5) Mutatu, W.; Klettke, K. L.; Foster, C.; Walker, K. D. *Biochemistry* **2007**, *46*, 9785–9794.
(6) Montavon, T. J.; Christianson, C. V.; Festin, G. M.; Shen, B.; Bruner, S. D. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3099–3102.
(7) Krug, D.; Mueller, R. *ChemBioChem* **2009**, *10*, 741–750.
(8) Szymanski, W.; Wu, B.; Weiner, B.; de Wildeman, S.; Feringa, B. L.; Janssen, D. B. *J. Org. Chem.* **2009**, *74*, 9152–9157.
(9) Fortin, P. D.; Walsh, C. T.; Magarvey, N. A. *Nature* **2007**, *448*, 824–827.
(10) Feng, L.; Wanninayake, U.; Strom, S.; Geiger, J.; Walker, K. D. *Biochemistry* **2011**, *50*, 2919–2930.
(11) Cooke, H. A.; Bruner, S. D. *Biopolymers* **2010**, *93*, 802–810.